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Distribution of the Intermedilysin Gene among the Anginosus Group Streptococci and Correlation between Intermedilysin Production and Deep-Seated Infection with *Streptococcus intermedius*

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The distribution of intermedilysin, a human-specific cytolysin, among the anginosus group streptococci and the correlation of toxin production and infection by *Streptococcus intermedius* were investigated. PCR and Southern hybridization specific for the intermedilysin gene revealed that the toxin gene exists only in *S. intermedius* and no homologue to the toxin gene is distributed in *S. anginosus* and *S. constellatus*. Thus, the intermedilysin gene is useful as a marker gene of *S. intermedius*. Moreover, a human-specific hemolysis assay and Western blotting with intermedilysin-specific antibodies clearly demonstrated that the intermedilysin production level in isolates from deep-seated infections, such as brain and liver abscesses, is higher (6.2- to 10.2-fold, respectively) than in strains from normal habitats, such as dental plaque, or from peripheral infection sites. However, other candidate virulence factors of *S. intermedius*, such as chondroitin sulfate depolymerase, hyaluronidase, and sialidase activities, did not show such a clear correlation between enzymatic activity and isolation sites or disease severity. From these results, intermedilysin is likely to be the pathogenic or triggering factor of significance in inducing deep-seated infections with *S. intermedius*.

Anginosus group streptococci, which form a part of normal human oral flora, are known to be pathogens in endogenous infections not only in the oral cavity but also at deep sites (12, 16). Of the three species within this group (2), *Streptococcus intermedius* is of particular interest since it shows a tropism for infections of the brain and liver and infection with this species is linked to abscess formation (16). *S. intermedius* produces many hydrolytic enzymes that include proteases (5) and glycosidases, such as sialidase (1) and hyaluronidase (4, 6), which destroy the host tissues and presumably convert them into small nutrient molecules to be utilized in bacterial growth and so may be important in the pathogenesis of infections with this species. Moreover, other bacterial products, such as an immunosuppressive protein (8) or an albumin-binding protein (18), have been reported as potential virulence factors. However, since no systematic experiments aimed at finding any correlation between bacterial products and infection have been carried out, their role(s) in streptococcal infection has still not been clarified. Recently, we reported that a human-specific cytolysin, intermedilysin (ILY), is secreted from a strain of *S. intermedius* that was isolated from a human liver abscess (9, 10). Because this toxin was shown to be able to directly damage host cells, including human cell lines derived from the organs likely to be invaded by *S. intermedius*, it is quite possible that this toxin contributes significantly to infections involving this species. In the present study, we reveal the distribution and expression level of the ILY gene among anginosus group streptococci isolated from various sites of infection, including its normal habitat, the human oral cavity. Moreover, we examine and discuss the pathogenic factors which may take key roles in deep-seated infection with *S. intermedius* by comparing the expression level of ILY with the activities of several glycosidases which are also recognized as candidate virulence factors.

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MATERIALS AND METHODS

Bacteria. The strains used in this study are listed in Table 1. All strains were identified phenotypically, and a subset of them were also genotypically classified at the species level according to methods described previously (14, 16). All strains were kept at −70°C and cultivated on blood agar containing 5% (vol/vol) defibrinated horse blood at 37°C in anaerobic conditions.

Hemolytic assay. Each strain was inoculated into 5 ml of brain heart infusion broth and cultured at 37°C for 18 h. The cells and supernatants were recovered by centrifugation at 3,500 rpm (3,500 × *g*) for 20 min in a bench-top centrifuge (type B and T; Searle Co.). Cells were washed once with Dulbecco's phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} (PBS contained 137 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4 \cdot 12H_2O$, and 1.5 mM KH_2PO_4; pH 7.4) and then once with distilled water, and then they were centrifuged, dried in an oven at 100°C overnight, and weighed. The culture supernatants were stored at −70°C until used. Hemolytic reactions were carried out in 1 ml of PBS containing 15 μl of 50% (vol/vol) erythrocyte suspension and 10 μl of the diluted culture supernatant at 37°C for 1 h, as described previously (9). The erythrocytes, of human, horse, and sheep origin, were prepared by washing them three times with PBS by centrifugation from blood stored in Alsever's solution. Subsequently, the reaction mixtures were centrifuged at 7,000 rpm (7,000 × *g*) in a Heraeus Biofuge 13 (Heraeus Sepatech) for 5 min. Three hundred microliters of each supernatant was dispensed into the wells of a 96-well microtiter plate, and absorbances at 540 nm were measured in a microtiter plate reader, Anthos Labtec HT3. After assaying a serial (3^n, $n = 3$–10) dilution series of the culture supernatants, the dilution factor of each supernatant that yielded half of the maximum (100%) hemolysis was calculated as described previously (9), and the reciprocal of the dilution factor was taken as the total number of hemolytic units in the supernatant. A hemolytic unit (U) was defined as the activity showing half of the maximum hemolysis of the hemolytic assay under the conditions described above. The hemolytic activity of the supernatant from each strain was expressed as the specific hemolytic activity (units per milligram [dry weight] of cells) in the results. A semiquantitative hemolytic assay on 5% (vol/vol) blood agar plates (agar depth = 4 mm) was carried out as follows. A single colony of each strain was picked up with a loop (diameter = 4 mm), the blood agar was inoculated by puncturing, and then plates were incubated at 37°C for 1 day anaerobically. Each hemolytic zone was traced onto tracing paper, and the area of the zone was measured by weighing.

TABLE 1. Strains of anginosus group streptococci used in this study

Isolation source	Strain(s) of:		
	S. intermedius	*S. anginosus*	*S. constellatus*
Head and neck			
Acute sinusitis	HARDY-DAVID T-1		
Aural swab			SL35
Brain abscess	2Q, 8F grey, 10a, 38, A4676a, CDC415/87, HW7, HW58, HW69, NMH2, UNS35	7k	
Craniotomy			HW24
Dental abscess	DP101, DP102	MS108	1769, DP10
Dental plaque	AC800, AC4629, AC4720, AC4730/S, AC5165, AC5803, AC7849, AM47, AM1524, AM2702, AM4902, AM8276, AM9256, EF491, EF1444, GN72, GN472, GN556, GN4623, GN6146, PC574, PC594, PC941, PC1483, PC2392, PC7466	EF222, PC4890	1340 (subgingival plaque), H251, T269, T338
Eye	E691		
Nasal swab			FW73
Oral pus			NMH12
Palatal abscess		NCTC11169	
Sputum		R84/4972	
Submandibular abscess			W862
Throat		I751large (β-gr. C),[a] NCTC10713	NCTC11063
Throat swab		3206336	3206443
Tooth socket			W277
Tracheostomy (stabbing)			W2/28
Pleuropulmonary			
Lung abscess			F436
Purulent pleurisy			NCDO2226
Musculoskeletal			
Osteomyelitis (spine)			C1792
Pelvic pus		HW44, NMH3 (β-gr. C)	
Blood		AS/JM4	NMH4, R87/3795
Abdominal			
Abdominal abscess	F600		
Abdominal mass	F458s	M5823 (β-gr. C)	W414
Appendix		48, SL28	47, 50
Appendectomy abscess			3206856
Inguinal hernia repair			3206797
Kidney swab		NMH5	
Liver abscess	27s, 32, 39, 40, 42, 43, 45, UNS46		34
Rectal abscess		KR533	
Rectal mucosalcolitis		138ii	303\
Umbilical	HW13		
Miscellaneous skins and soft tissues			
Arm abscess	F44R		
Axillary abscess		KR687	
Bite wound, hand	WS100s		
Fistula (dog)		Str778	
Inguinal abscess			NMH6
Leg ulcer		3212659, R87/1657	
Leg wound			W207
Perianal abscess		HW31	
Pilonidal abscess	3206393[b]	M6591	
Pilonidal sinus abscess anus	3206692[b]		
Toe ulcer			3212472
Urogenital			
Bartholin abscess		PH10-11	
Urine		KR461	
Uterus pus			HW74
Vagina		POOLE PHLS 457, R88/111, W896	
Unknown	M6873 (surgical abscess), NCDO2227, NMH8 (wound swab)		

[a] β-gr. C, β-hemolytic Lancefield group C strains (17).
[b] Atypical *S. intermedius* strains (15, 17; Whiley and Hardie, unpublished data).

PCR of the ILY gene. Preparation of the bacterial genomic DNA was performed as follows. Streptococcal cells cultured in 20 ml of brain heart infusion broth supplemented with 5% (vol/vol) horse serum were harvested by centrifugation and washed with 0.8 ml of TE (10 mM Tris-HCl, 1 mM EDTA; pH 8.0). The washed cells were suspended in 0.1 ml of TE and mixed with 10 μl of 50-mg/ml lysozyme in TE. The enzyme reaction was allowed to proceed at 37°C for 30 min, and then 0.5 ml of the lysis mixture containing 60% (wt/vol) guanidine isothiocyanate, 100 mM EDTA sodium salt (pH 8.0), and 0.5% (wt/vol) *N*-laurylsarcosine was added to each reaction mixture. The mixture was mixed and left at room temperature for 10 min to complete cell lysis. After the addition of 0.25 ml of cold 7.5 M ammonium acetate, the mixture was mixed and placed on ice for 10 min. Subsequently, 0.5 ml of chloroform-isoamylalcohol (24:1) was mixed thoroughly with each reaction mixture, and the mixture was centrifuged at 13,000 rpm (15,000 × *g*) for 10 min in a microcentrifuge, Heraeus Biofuge 13. Seven hundred microliters of each supernatant was transferred to a new microcentrifuge tube and mixed with 0.378 ml of cold isopropanol. After gently mixing by inversion of the tube for 1 min, the mixture was centrifuged at 13,000 rpm (15,000 × *g*) for 2 min and the supernatant was discarded. Precipitated DNA was resuspended in 0.2 ml of TE. After the addition of 0.5 ml of cold absolute ethanol to the DNA solution, the mixture was placed at −70°C for 1 h and then centrifuged at 13,000 rpm (15,000 × *g*) for 3 min. Subsequently, the supernatant was removed and the precipitated DNA was air dried for 30 min. Finally, the DNA was dissolved in 0.1 to 0.3 ml of TE and stored at −70°C, ready for use. PCR amplification of the partial ILY gene was carried out in a reaction volume of 50 μl containing 1 μl of the template genomic DNA solution, 0.25 mM concentrations of each deoxyribonucleoside triphosphate (Amersham Pharmacia Biotech), 1 U of *Taq* DNA polymerase (Amersham Pharmacia Biotech), 20 pmol of ILY-NFw (5′-AACACCTACCAAACCAAAAGCAGC-3′), 20 pmol of ILY-CBw (5′-ACTGTGGATGAAGGGTTGTTCCCC-3′), 50 mM KCl, 10 mM Tris-HCl (pH 8.8) and 1.5 mM $MgCl_2$. The PCR program was as follows: initial denaturation at 95°C for 5 min, followed by 30 cycles of a denaturation step at 95°C for 1 min, an annealing step at 55°C for 1 min, and an extension step at 72°C for 2 min. The expected amplified fragment size was 1,463 bp of the coding region of the ILY gene. The PCR products were analyzed by 0.8% agarose gel electrophoresis in a TBE (71 mM Tris, 89 mM boric acid, 2 mM EDTA; pH 8.0) buffer system. To confirm the origin of the amplicons, digestion with *Nde*I was carried out, which cleaved the amplicon from the ILY gene into two fragments of 796 and 667 bp. To confirm the presence and integrity of the genomic DNA, the amplification of the 16S-23S rRNA gene spacer was carried out on all DNA samples tested according to the method of Whiley et al. (15).

Southern blotting of the ILY gene. Genomic DNAs extracted from the type strains of *S. anginosus* (NCTC10713), *S. constellatus* (NCDO2226), and *S. intermedius* (NCDO2227) and from *S. intermedius* UNS46 were treated with RNase, digested by *Eco*RI, and electrophoresed in 1% agarose gels. After confirmation of the fragmentation of genomic DNA by staining with ethidium bromide, the DNA fragments were transblotted onto a GeneScreen Plus membrane (NEN) according to the standard method (13). Hybridization was carried out with a ^{32}P-labelled ILY gene fragment of 474 bp amplified by PCR with primer set A (5′-TCTCAGCTGCATTCGCTGAAACACC-3′ and 5′-TAACAGCTGTTCG AACTGTA-3′) as the N-terminal side probe and a fragment of 756 bp amplified with primer set B (5′-GCCGTGCAATGTATGTGAAA-3′ and 5′-CGAAGAT TCAAGGCTTCTCTCGG-3′) as the C-terminal side probe. Blotted membranes were prehybridized at 68°C for 1 h in 6 × SSC containing 5% (wt/vol) skimmed milk, 0.02% (wt/vol) sodium azide, and 1% (wt/vol) sodium dodecyl sulfate (SDS) (1 × SSC contains 150 mM NaCl and 15 mM sodium citrate [pH 7.5]). Each probe (final activity, approximately 5×10^5 cpm/ml) and 0.2 mg of salmon sperm DNA were added to 0.5 ml of distilled water, heat treated at 95°C for 3 min, and immediately chilled. After the prehybridization, each probe solution was added to the 6 × SSC prehybridization solution and hybridized with the genomic DNA at 68°C overnight. Subsequently, the membranes were washed twice with 2 × SSC containing 1% SDS at room temperature for 5 min and then washed with 0.2 × SSC containing 1% SDS at 68°C for 30 min. Dried membranes were exposed to X-ray films at −80°C overnight.

Antibodies against ILY. A linear peptide of the N-terminal side of ILY that was attached with a cysteine residue on the carboxyl terminus (Y^{73}DKLNILT HQGEKLKNHSS^{91}C, N-terminal side peptide) and a linear peptide of the C terminus of ILY that were attached with a cysteine residue on the amino terminus (CW^{514}GTTLHPQFEDKVVKDNTD^{532}, C terminus peptide) were synthesized by fluorenylmethoxycarbonyl chemistry in a Shimadzu PSSM-8 peptide synthesizer, as described previously (9). After the purification of both peptides by reverse-phase liquid chromatography, 4 mg of each peptide was conjugated with 8 mg of *N*-ethylmaleimide-pretreated keyhole limpet hemocyanine (KLH) activated with *N*-(γ-maleimidobutyryloxy)succinimide. The N-terminal side peptide-conjugated antigen was used with Freund's complete adjuvant (0.5 mg of peptide bound to KLH/mouse) to immunize mice intraperitoneally. After 2 weeks, mice were given boosters of the antigen solution containing 0.4 mg of peptide bound to KLH emulsified with Freund's incomplete adjuvant, intraperitoneally. Finally, the mouse antibodies were boosted with the antigen solution containing the same amount of peptide 2 weeks after the last booster, intravenously. Three days after the final booster, the splenocytes from the immunized mice were hybridized with a myeloma cell line, SP2/0-Ag14, by using polyethyleneglycol 4000 and then cultured in hypoxanthine-aminopterin-thymidine medium according to a standard method (7). The hybridomas secreting anti-N-terminal side peptide monoclonal antibodies (MAbs) were screened by an enzyme immunoassay in microtiter plates coated with the N-terminal side peptide (1 μg/well). The selected hybridomas were cloned by limiting dilution twice and were thus established. The polyclonal antibody against the C terminus peptide was raised in rabbits as described previously (9). The amounts of antigen used in the primary and booster immunizations were 0.5 and 0.25 mg of peptide bound to KLH, respectively. Finally, the immunoglobulin G (IgG) fraction of the anti-C terminus peptide serum was purified by protein A affinity chromatography.

Western blotting. Each 10-fold-condensed supernatant of the anginosus group isolates, concentrated by lyophilization, was applied to Laemmli's SDS-polyacrylamide gel electrophoresis and transblotted onto hydrophobic polyvinylidene difluoride membranes. After rinsing with deionized water three times, the blotted membranes were blocked with PBS containing 5% (wt/vol) bovine serum albumin (BSA) for 1 h. The membranes were dipped into the first antibody solution (the culture supernatant of hybridoma which contained MAb INTN-91 or the 1.0 μg of anti-C terminus peptide rabbit IgG per ml of PBS containing 1% BSA) for 1 h. Then, the membranes were washed once with PBS for 5 min and three times with PBS containing 0.1% (wt/vol) Tween 20 for 5 min. Subsequently, the membranes were reacted with horseradish peroxidase-labelled anti-mouse IgG or anti-rabbit IgG antibody diluted in PBS containing 1% BSA for 1 h. The membranes were washed as described above. Finally, the membranes were dipped into the substrate solution containing 0.05% (wt/vol) diaminobenzidine and 0.02% (vol/vol) H_2O_2 to allow the development of a positive signal. All immunoblotting processes were carried out at room temperature.

Enzyme assay. Bacterial hyaluronidase and chondroitin sulfate depolymerase activities were spectrophotometrically measured with sodium hyaluronidate from human umbilical cord (Sigma H-1876) and sodium chondroitin sulfate A (Sigma C-8529) from bovine trachea as the substrates according to the methods of Homer et al. (4), with slight modifications. The absorbance changes of Stains-All, indicating the decrease of the substrates, were measured at 620 and 450 nm, respectively, in a microtiter plate reader (Anthos Labtec HT3). Sialidase assays were carried out according to the method of Beighton and Whiley (1), and any reaction product was fluorophotometrically measured in a Millipore Cytofluor 2300 (excitation, 360 nm; emission, 460 nm). Activities of hyaluronidase and sialidase were estimated with the bacterial suspension (0.1 absorbance at 620 nm, approximately 10^8 organisms/ml) as the samples. Chondroitin sulfate depolymerase activity was estimated after the 4-day cultivation of the bacteria in the test mixture.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases with the accession no. AB029317.

RESULTS

Detection of the ILY gene by PCR and Southern hybridization in the Anginosus group streptococci. Figure 1 shows the PCR amplification product of the ILY gene in anginosus group streptococci. Fifty-seven out of 59 strains (97%) of *S. intermedius*, including the type strain, were positive, i.e., they produced a 1,463-bp amplicon, but no DNA fragment was amplified from the genomic DNA from all the strains (0%) of *S. anginosus* ($n = 29$) and *S. constellatus* ($n = 31$) tested. Furthermore, digestion of all amplicons with *Nde*I produced two bands, 0.8 and 0.7 kbp (data not shown), indicating that all amplicons were derived from the ILY gene. Both of the PCR-negative *S. intermedius* strains (3206393 and 3206692) in fact showed atypical phenotypes and genotypes, demonstrating that they should be excluded from the *S. intermedius* species (17; R. A. Whiley and J. M. Hardie, unpublished data). The results of genomic Southern hybridization of the ILY gene with the three species and the N-terminal and C-terminal side probes are shown in Fig. 2. The presence of the genomic fragment carrying the ILY gene was clearly demonstrated with the *S. intermedius* type strain, NCDO2227, as well as with UNS46, as one band, but no signal was found with the *S. anginosus* and *S. constellatus* type strains. These results demonstrate that within the anginosus group streptococci, the ILY gene is found only in *S. intermedius* and that the gene is present in all *S. intermedius* strains studied thus far. Moreover, no toxin gene closely related to the ILY gene seems to exist in *S. anginosus* or *S. constellatus*.

Expression of the ILY gene in the anginosus group streptococci. Firstly, the expression of the ILY gene in all *S. interme-*

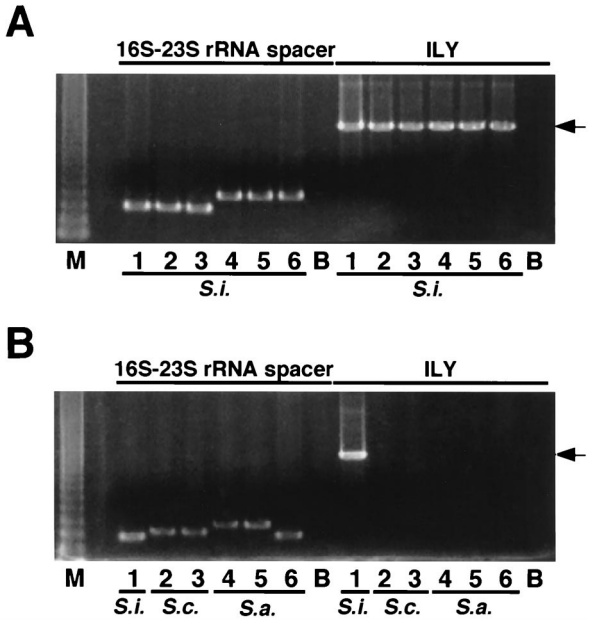

FIG. 1. Amplification of the ILY gene in anginosus group streptococci by PCR. (A) *S. intermedius* (*S.i.*) strains. Lanes 1, NCDO2227^T; lanes 2, UNS46; lanes 3, 40; lanes 4, 45; lanes 5, 39; lanes 6, 8F grey. (B) *S. anginosus* (*S.a.*) and *S. constellatus* (*S.c.*) strains. Lanes 1, *S. intermedius* NCDO2227^T as a positive control of PCR amplification; lanes 2, NCDO2226^T; lanes 3, 3206856; lanes 4, NCTC10713^T; lanes 5, NCTC11169; lanes 6, M6591. Lanes M, 123-bp ladder marker; lanes B, no template DNA, i.e., negative control; ILY, results of the amplification of the ILY gene fragment (arrows indicate the 1,463-bp fragment); 16S-23S rRNA spacer, results of the amplification of the 16S-23S rRNA gene spacer as a monitor for genomic DNA integrity.

dius strains was determined by using ILY-specific MAb INTN-91, which recognizes the N-terminal side of ILY, and a polyclonal antibody against the C terminus of ILY. Figure 3 shows the typical Western blotting pattern obtained. The same 54-kDa molecule of ILY, which is the mature size of the toxin, was detectable in each culture supernatant of all strains of *S. intermedius* by both antibodies, whereas the production level of ILY varied between strains. Of particular note was that significantly more ILY was produced by strains from deep-seated infections, such as brain and liver abscesses, than by strains isolated from the normal habitat, dental plaque. ILY production in strains from peripheral sites of infection (such as the eye, arm abscess, or sinuses) was also weak and showed essentially the same range of activities as those from dental plaque strains (data not shown). No ILY was found in the culture supernatants from the ILY gene-negative atypical *S. intermedius* strains, 3206393 and 3206692, or from any strains of *S. anginosus* and *S. constellatus* (data not shown).

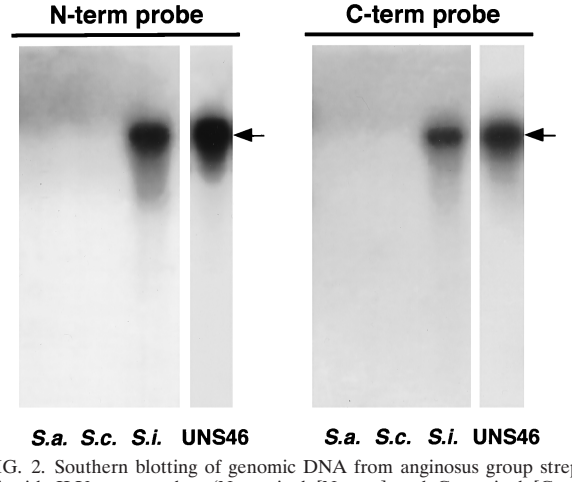

FIG. 2. Southern blotting of genomic DNA from anginosus group streptococci with ILY gene probes (N-terminal [N-term] and C-terminal [C-term] probes). *S.a.*, *S. anginosus* NCTC10713^T; *S.c.*, *S. constellatus* NCDO2226^T; *S.i.*, *S. intermedius* NCDO2227^T; UNS46, *S. intermedius* UNS46. Arrows indicate the fragment carrying the ILY gene (16 kbp).

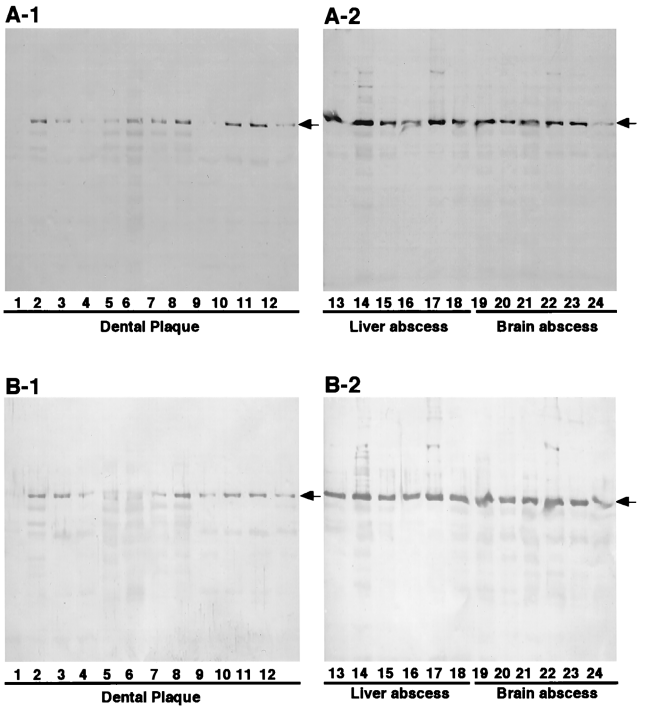

FIG. 3. Western blotting of ILY in culture supernatants of *S. intermedius* strains. (A-1 and A-2) Results for dental plaque strains and the type strain (A-1) and liver and brain abscess strains (A-2) with MAb INTN-91 as the ILY probe. (B-1 and B-2) Results for the same strains as in A-1 and A-2 but with an anti-C-terminus peptide rabbit IgG as the probe instead. Lanes 1, NCDO2227; lanes 2, PC1483; lanes 3, PC2392; lanes 4, AC800; lanes 5, AC4730/S; lanes 6, AC5803; lanes 7, AC5165; lanes 8, EF1444; lanes 9, AM8276; lanes 10, GN4623; lanes 11, EF491; lanes 12, AM4902; lanes 13, 32; lanes 14, 40; lanes 15, 42; lanes 16, 43; lanes 17, UNS46; lanes 18, 27s; lanes 19, NMH2; lanes 20, UNS35; lanes 21, 8F grey; lanes 22, A4676a; lanes 23, 38; lanes 24, HW58. Arrows indicate the mature ILY (54 kDa).

In order to quantitatively compare ILY production among strains of *S. intermedius*, we quantified the ILY activity in each culture supernatant by a hemolytic assay with human, sheep, and horse erythrocytes. In all culture supernatants from 57 strains of typical *S. intermedius*, hemolysin activity was detected with human erythrocytes. Most hemolytic activities found in the supernatants were human specific, i.e., ILY activity. As shown in Fig. 4A, ILY production by the strains from brain abscesses and abdominal infections was obviously higher than that found with strains from dental plaque. The average human-specific hemolytic activities shown by the brain abscess and abdominal infection strains were 3,900 ± 3,670 and 2,376 ± 2,760 (mean ± standard deviation [SD]) U/mg [dry weight] of cells, respectively. These values are approximately 10.2- and 6.2-fold higher than those of dental plaque strains (381 ± 185 U/mg [dry weight] of cells), respectively. A statistically significant difference was found between the average hemolytic activities of the strains from these deep infection sites and that of the dental plaque strains. The mean ILY

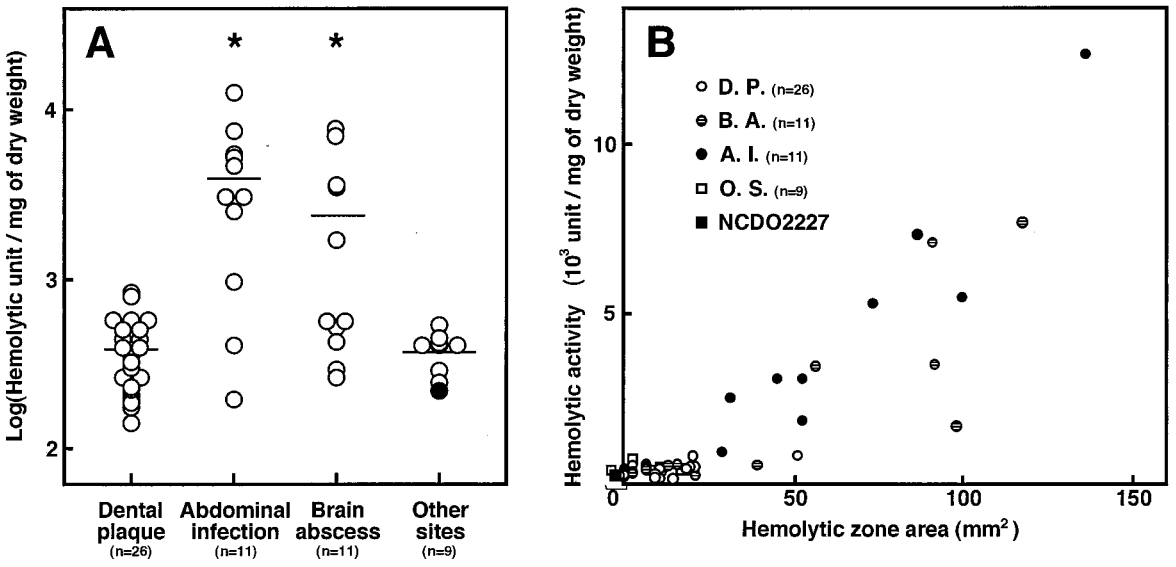

FIG. 4. ILY activity in the culture supernatants of *S. intermedius* strains and the correlation between the ILY activity and hemolytic zone area in human blood agar. (A) Hemolytic activity in the culture supernatants of *S. intermedius* strains measured in the tube hemolysis assay. Bars indicate the mean of the hemolytic activity of each group. Closed circle, NCDO2227, the type strain of *S. intermedius*. Asterisks indicate a significant difference of the means of hemolytic activities between strains from each isolation site and strains from the normal habitat, dental plaque, by Student's *t* test ($P < 0.01$). (B) Correlation between the hemolytic activity and the hemolytic zone area produced by each *S. intermedius* strain. D. P., dental plaque group; B. A., brain abscess group; A. I., abdominal infection group; O. S., other sites group. Hemolytic zone areas of the strains on the vertical axis and outside of the frame were zero.

activity in the strains from other peripheral sites, except for the two atypical stains, was 376 ± 105 U/mg [dry weight] of cells and was comparable to that of the dental plaque strains (no significant difference was found by using Student's *t* test). Moreover, we tested the ILY production of each *S. intermedius* strain on human or horse blood agar containing host human or horse blood components. As shown in Fig. 4B, a strong positive correlation was found between hemolytic activity and the hemolysis zone size obtained with human blood plates among strains from brain abscesses ($r = 0.829$) and abdominal infections ($r = 0.940$), respectively. On the other hand, the correlation was less significant among dental plaque strains ($r = 0.676$) and those strains from other peripheral sites ($r = 0.337$). Among strains from dental plaque, most were weak producers of ILY, even in the presence or absence of human plasma components. However, there was an exception (strain EF491) which can express high levels of ILY and cause human-specific hemolysis on human blood plates. Of the 59 strains tested, 46 (78%) formed a human-specific hemolytic zone, but 13 (22%), AC4629, AM47, AM1524, AM2702, AM8276, DP102, HW69, PC574, PC594, PC941, 45, 3206393, and 3206692, also showed hemolytic zones on horse blood agar. The hemolytic zone areas on the human and horse blood agars caused by each of 12 of these strains (mean ± SD, 12.0 ± 4.6 mm^2) were essentially the same. The exception was strain 45, which yielded a 10-fold-larger area on human blood agar than on horse blood agar. Interestingly, however, the hemolysis of human erythrocytes induced by hemolysin (presumably ILY) which was secreted by *S. intermedius* into the culture supernatant was easily detected in the standard tube assay as described in Materials and Methods, but non-human-specific hemolytic activity found in the above-mentioned 13 strains by the blood agar method could hardly be determined in the standard tube assay system. The nonspecific activity in those strains was at most 20 U/mg [dry weight] of cells in the standard tube hemolytic assay with sheep or horse erythrocytes. Nonspecific hemolysis was found in 30% of the strains from dental plaque or from peripheral infection sites, i.e., 3.3-fold more frequently than among strains from deep infection sites (9.1%). We also tested 29 strains of *S. anginosus* and 31 strains of *S. constellatus*. Nine strains (31%) of *S. anginosus* and 18 strains (58%) of *S. constellatus* were hemolytic on both blood agars. The hemolytic zone area tended to be slightly larger (at most 2-fold) on horse blood agar than on human blood agar (data not shown). However, no human-specific hemolysis by any strains of these two species was detected. These results are consistent with those from the PCR detection, genomic Southern hybridization, and Western blotting of ILY with *S. anginosus* and *S. constellatus*. The hemolytic activities of the *S. anginosus* and *S. constellatus* strains were also hardly detectable in the standard hemolytic assay system, even in the presence of 10 mM dithiothreitol. The same applied to the nonspecific activity shown by 13 strains of *S. intermedius*.

Glycosidase activities in *S. intermedius* strains. We randomly selected a subset of *S. intermedius* strains from dental plaque ($n = 10$) and deep infection sites, including the brain ($n = 11$) and liver ($n = 8$), together with *S. intermedius* type strain NCDO2227, to compare several glycosidase activities among these three groups. Enzymes such as hyaluronidase, chondroitin sulfate depolymerase, and sialidase are abundantly produced by *S. intermedius* and constitute factors which can cause the host tissue damage and introduce deep-seated infections (1, 4, 6, 16). We hypothesized that, if these glycosidases play a key role in such infections by *S. intermedius*, an obvious correlation between the clinical isolation site(s) and enzyme activity(ies) might be observed. Figure 5 shows the activities of hyaluronidase, chondroitin sulfate depolymerase and sialidase of these strains. The average hyaluronidase activities of the dental plaque, brain abscess, and liver abscess strains were 1.00 ± 0.14, 0.99 ± 0.16, and 0.90 ± 0.22 (mean ± SD) μg of substrate hydrolyzed/h, respectively; the activity of the type strain, NCDO2227, was 1.00 μg/h. The average chondroitin sulfate depolymerase activities of the dental plaque, brain abscess, and liver abscess strains were 153 ± 14.0, 151 ± 9.00, and 157 ± 7.33 (mean ± SD) μg of substrate hydrolyzed/4 days, respectively; the activity of the type strain was 151 μg/4 days.

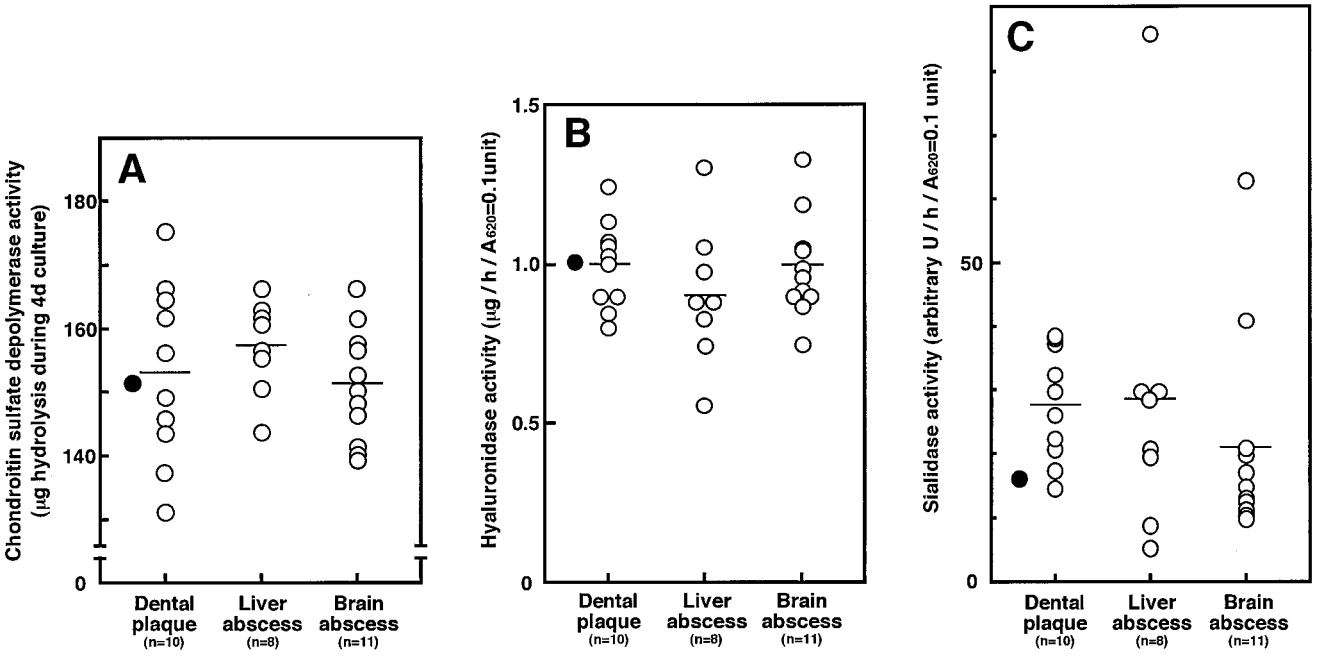

FIG. 5. Glycosidase activities of *S. intermedius* strains from dental plaque and abscesses. (A) Chondroitin sulfate depolymerase activity; (B) hyaluronidase activity; (C) sialidase activity. Bars indicate the mean value of the activity of each group. Closed circles indicate the activity of the type strain, NCDO2227.

Finally, the average sialidase activities of the dental plaque, brain abscess, and liver abscess strains were 27.7 ± 8.84, 21.2 ± 16.4, and 28.5 ± 25.0 (mean ± SD) arbitrary units of the substrate hydrolyzed/h, respectively; the type strain showed an activity of 16.2 arbitrary units/h. As shown previously (14, 16), these enzymatic activities were high in all strains of *S. intermedius*, and there were no significant differences in the hyaluronidase and chondroitin sulfate depolymerase activities between the three clinical site groups, whereas sialidase activities varied much more between strains.

DISCUSSION

The human-specific cytolysin ILY was previously found to be produced by an *S. intermedius* strain from a human liver abscess (9, 10). However, the distribution of ILY production not only in *S. intermedius* strains but also among strains of the whole anginosus group of streptococci, i.e., *S. intermedius*, *S. anginosus*, and *S. constellatus*, has not yet been determined. In addition, it is important to understand the infection mechanism of *S. intermedius* and the role that ILY has to play. Therefore, we examined the distribution of the ILY gene and its expression among the anginosus group of streptococci.

Using a PCR primer set to amplify the region encoding most of the mature ILY molecule, we detected the gene in all strains of *S. intermedius* except for two that were phenotypically and genetically atypical (15, 17). If an anginosus group species possesses a toxin(s) very similar to ILY, as is the case for the pyogenic species group (i.e., *S. pyogenes*, *S. canis*, and *S. equisimilis*, of which the latter two species produce toxins that are strikingly similar to streptolysin O [11]), then a gene fragment having homology to the ILY gene would be expected to be amplified by PCR or a gene homologue of the ILY gene would be expected to be detected by genomic Southern hybridization in *S. anginosus* and *S. constellatus*. However, no specific gene amplicon or hybrid was found within the other anginosus group streptococci. These results revealed that the ILY gene is present throughout *S. intermedius* and that a molecule closely related to ILY is not distributed in *S. anginosus* or *S. constellatus*. The ILY gene seems to be derived from the same ancestor as so-called "cholesterol-binding cytolysins" or "thiol-activated cytolysins" (H. Nagamune et al., unpublished data). Among the six species groups within the genus *Streptococcus*, such cytolysins have been reported in three groups: pyogenic, mitis, and anginosus. In the pyogenic group in particular, cytolysins are thought to be widely distributed among the species within the group. However, from the present results, the homologue of the cholesterol-binding cytolysin has been inherited only by the *S. intermedius* species, within the anginosus group.

Subsequently, in order to gain some idea of the importance of ILY in *S. intermedius* infections, we examined the relationship between ILY expression and the severity of infections with this species. Having established that all strains of *S. intermedius* secrete mature ILY and that no strain of *S. anginosus* and *S. constellatus* produces ILY, we can say that the mean level of ILY in the culture supernatants of strains from deep sites, such as brain and liver abscesses, is much higher than that formed with strains from dental plaque or peripheral-site infections. This trend was also supported by the results of quantitative hemolytic assays of culture supernatants (Fig. 4A). The hemolytic assay in PBS with human, horse, and sheep erythrocytes revealed that strong cytolytic activity was detectable only in the culture supernatants of *S. intermedius*, that all strains identified as typical *S. intermedius* showed hemolysis of human erythrocytes in the standard assay, and that most of the activities detected among *S. intermedius* strains are indeed human specific. Such a correlation was also found in the human blood agar assay carried out in parallel (Fig. 4B). As shown in Fig. 5, the lack of significant correlation between other enzymatic activities (chondroitin sulfate depolymerase, hyaluronidase, and sialidase) and the infection sites strongly suggests that ILY is of importance, perhaps as a primary, or triggering, pathogenic factor, to ultimately cause deep-seated infections, such as

brain and liver abscesses caused by *S. intermedius*. Interestingly, there was one exception, dental plaque strain EF491, which produced high levels of ILY when cultured on human blood agar. The ILY gene in strain UNS46, a strong ILY-producing strain, has been shown to be present as a single copy (H. Nagamune et al., unpublished data), which is the same as for the type strain NCDO2227, which is a weakly ILY-producing strain, as shown in Fig. 2. Therefore, although we cannot yet present a clear picture of the regulation mechanism of ILY expression, it is quite possible that there are two types of strains: weak (or poorly inducible) ILY producers or strong (or potentially strongly inducible) ILY producers within *S. intermedius*. We postulate that if an individual carrying a strongly ILY-producing strain in their dental plaque experiences trauma within the oral cavity, the strain may enter the bloodstream and more readily cause a deep-seated infection. Conversely, if a weakly ILY-producing strain (represented by most *S. intermedius* strains, in our experience) enters the bloodstream, it is more easily removed by phagocytes, such as neutrophils or macrophages. However, since ILY has been observed to lyse cells of the human immune system, such as neutrophils (M. Taylor and R. A. Whiley [St. Bartholomew's and the Royal London School of Medicine and Dentistry], personal communication), we are postulating that ILY may function as an "escape factor" which lyses phagocytes that function to clear the strain from the blood, thereby enabling or aiding the strain to reach a deep anatomical site, fix onto tissue by some specific affinity mechanism, and eventually form an abscess, facilitated throughout the process by ILY, chondroitin sulfate depolymerase, hyaluronidase, and sialidase as tissue invasion factors. We cannot specify the factor(s) responsible for the organ tropism of the *S. intermedius* so far, although several potential tissue adhesion factors, such as the selection receptor [sialyl Lewis (x) antigen]-like glycocomponents which are abundantly expressed on the *S. intermedius* cell surface and cell surface hemagglutinin, have been reported (3, 19). In order to clarify the mechanism(s) of infection by *S. intermedius*, further investigation of the action mechanism of ILY and analysis of tissue tropisms are necessary. Moreover, to evaluate this working hypothesis, we may have to perform a histopathological study which reflects the in vivo reaction between the host and *S. intermedius* in deep-seated infections by this bacterium because we have only in vitro evidence that supports this hypothesis thus far. Studies on the interaction between *S. intermedius* and host cells, such as phagocytes and human cells derived from target organs with an ILY gene knockout mutant of the strong ILY producer strain, are currently proceeding.

As shown in the results, a rapid and reliable way to identify *S. intermedius* strains among anginosus group streptococci is by PCR amplification of the ILY gene as a marker. The nonspecific hemolytic activity found on blood agar with some *S. intermedius* strains is also of interest. Since this activity was difficult to estimate in the tube assay system, which is suitable for the detection of ILY or cholesterol-binding cytolysins (in the presence of dithiothreitol), the hemolysin responsible for the nonspecific activity seems to be essentially a completely different molecule from ILY or from cholesterol-binding cytolysins rather than an ILY-related toxin which has lost human specificity by some mutation. The hemolysin(s) activity which is nonspecific and distributed among certain strains of *S. anginosus* and *S. constellatus* is also difficult to detect in the tube assay system, even in the presence of dithiothreitol to activate cholesterol-binding cytolysins. Judging from the similarity in characteristics and the lacking of a gene related to the ILY or cholesterol-binding cytolysin genes in *S. anginosus* and *S. constellatus*, both these hemolysins may belong to another toxin group, such as streptolysin S or phospholipase.

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